

SUPPLEMENTAL DATA

Figure S1. Repeat of Fig. 1B in H1 and the single cell RT-PCR in H9.

- A. RT-qPCR repeat for markers listed in Fig.1B for another cell line H1.
- B. Correlation between gene expression levels measured in H9 and H1 for marker genes at indicated time points.
- C. Quantification of data in Fig. 1C. To quantify the differentiation efficiency, three to five fields were randomly selected. n = 3 independent experiments; two-tailed *t* test. All data are presented as the mean ± SD.
- D. Single cell RT-PCR analysis for OCT6, SIX3 and SOX1 at indicated time points.

Figure S2. Quality control and Principle component analysis (PCA) of the RNA-Seq Data.

- A. Number of genes detected in each sample.
- B. Boxplots showing the distribution of relative gene expression (median FPKM of all genes within a given module) for different time points.
- C. Hierarchical clustering analysis, under the red line (height = 80), as a cutoff, the neural differentiation process can be divided in more detail.
- D. Three dimensional PCA of transcriptomes of all samples at 12 different time points.
- E. The expression of genes with highest loadings in the first principal component, top 100 PC1 negative and positive genes with highest quantitative correlation are used to draw heatmap. Boxplots showing the distribution of genes expression for different time points.

Figure S3. Expression pattern of putative substage specific markers from RNA-Seq data.

Figure S4. Qualification analysis of potential markers across the neural differentiation process.

- A. Comparison of Expression patterns of potential marker genes with well-known genes in each module.
- B. Potential marker expression patterns in each module as revealed by RT-qPCR analysis (Fig.S4A).

Figure S5. Functional enrichment of TFs within each module

- A-E. GO analysis of biological process of TFs within each module.

Figure S6. Detection of pluripotency markers in the store culture of Control and CRISPR/Cas9 knockout cell lines.

- A. Immunostaining of pluripotency marker *OCT4*, *NANOG*, *TRA-1-81* and *SSEA3/4*. Scale bars, 75 µm.
- B. Quantification of the data in A. To quantify the differentiation efficiency, three to five fields were randomly selected. n = 3 independent experiments; two-tailed *t* test. All data are presented as the mean ± SD. *p < 0.05, **p < 0.01.

Figure S7. Immunostaining of the second clones of *SIX3* and *HESX1* KO cell lines.

- A. Double immunocytochemistry analysis of *OCT4* and *SOX2*, *OTX2* and *SOX2* in *SIX3* and *HESX1* KO cell lines on day 6.
- B. Quantification of the immunocytochemistry in A.
- C. Immunocytochemistry analysis of *PAX6* in *SIX3* and *HESX1* KO cell lines on day 12.
- D. Quantification of the immunocytochemistry in C.

E. Immunocytochemistry analysis of *NEUN* in SIX3 and HESX1 KO cell lines on day 50. Scale bars, 75 μ m (A, C, E).

F. Quantification of the immunocytochemistry in F. To quantify the differentiation efficiency, three to five fields were randomly selected. n = 3 independent experiments; two-tailed *t* test. All data are presented as the mean \pm SD.
*p < 0.05, **p < 0.01.

Table S1.

Supplemental Table 1. Transcription factors in each module.

Table S2.

Supplemental Table 2. SgRNA sequence.

Table S3.

Supplemental Table 3. Antibodies.

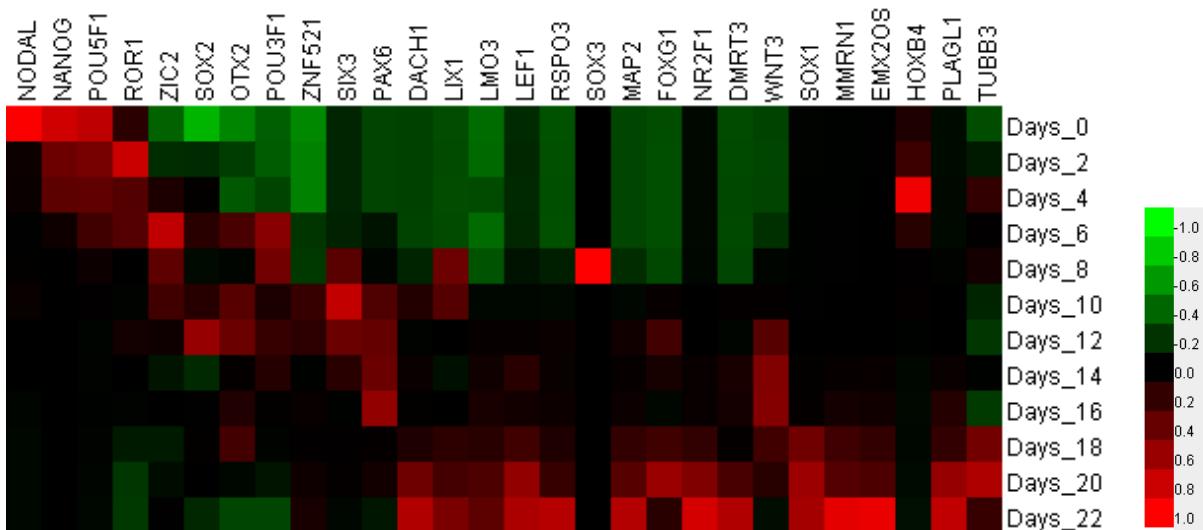
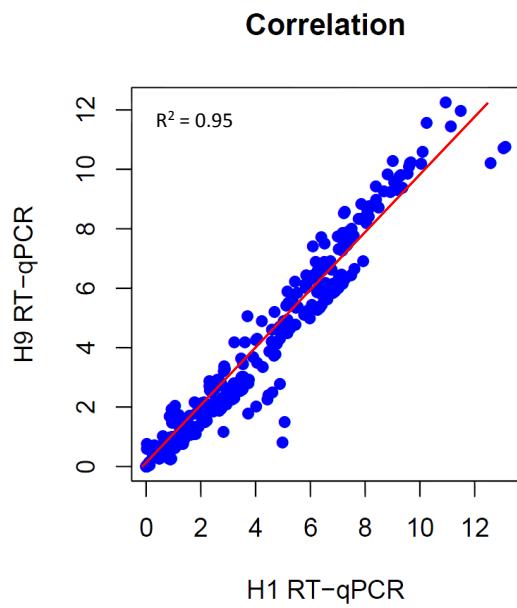
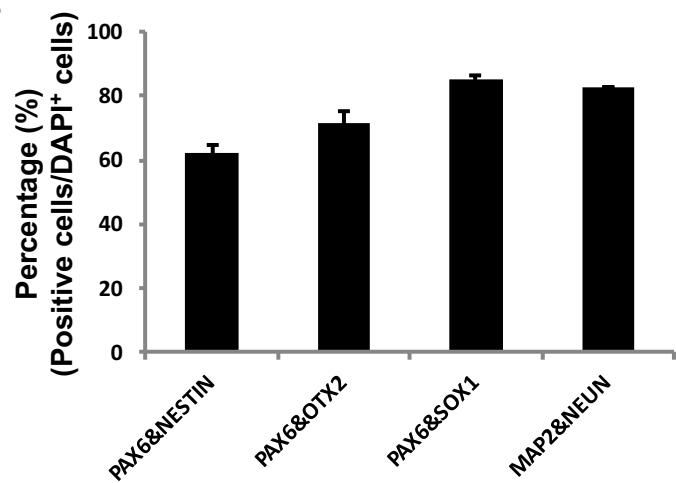
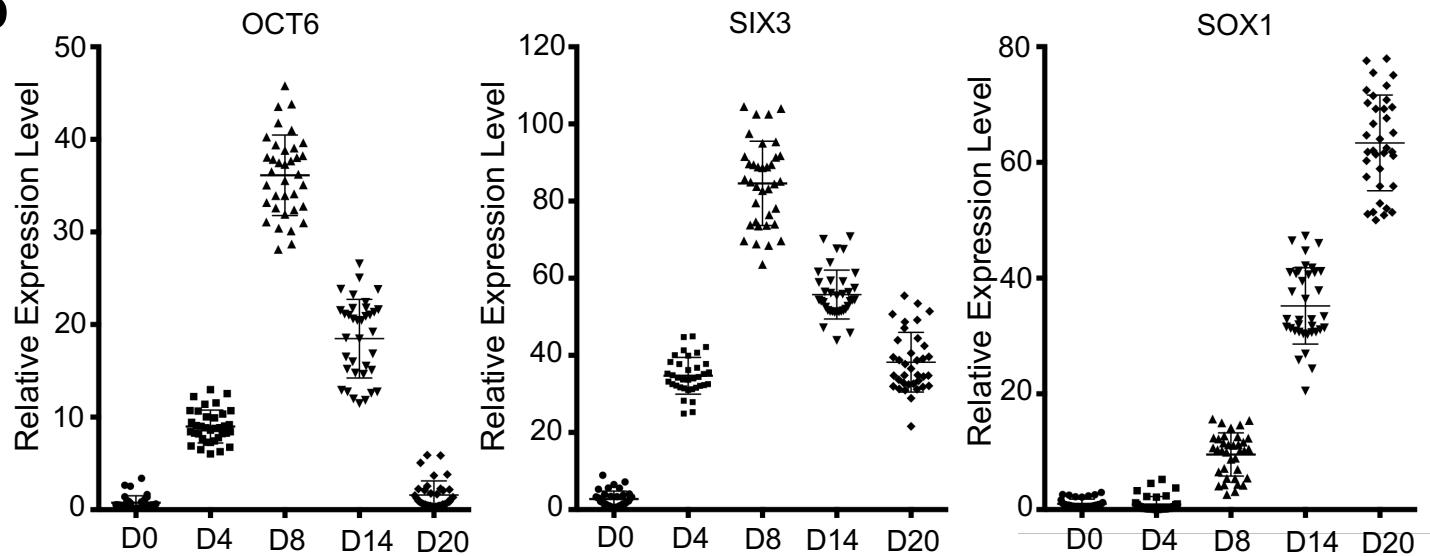
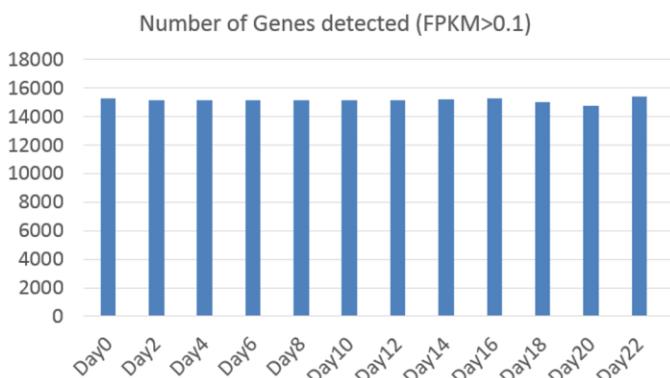
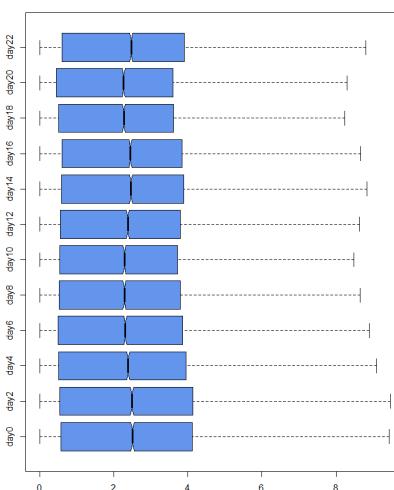
Figure S1**A****B****C****D**

Figure S2

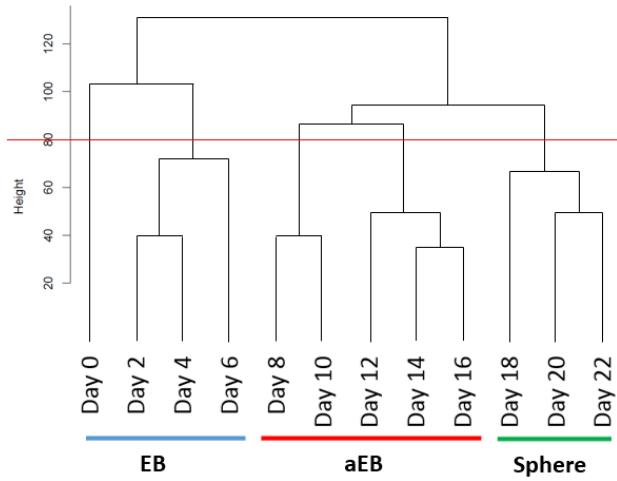
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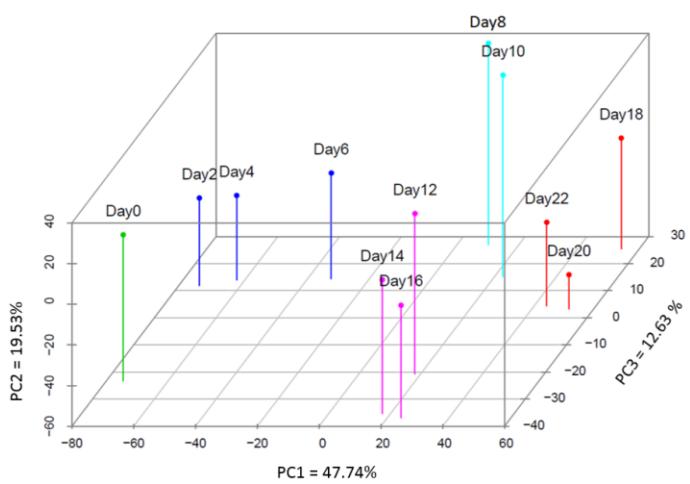
B



C



D



E

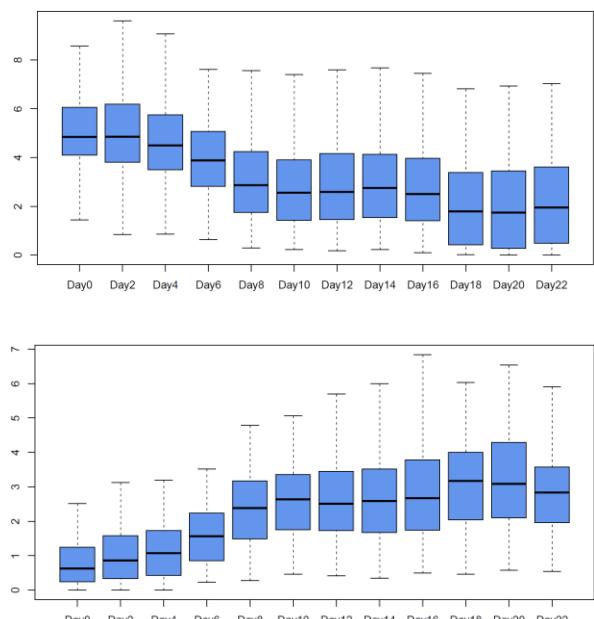
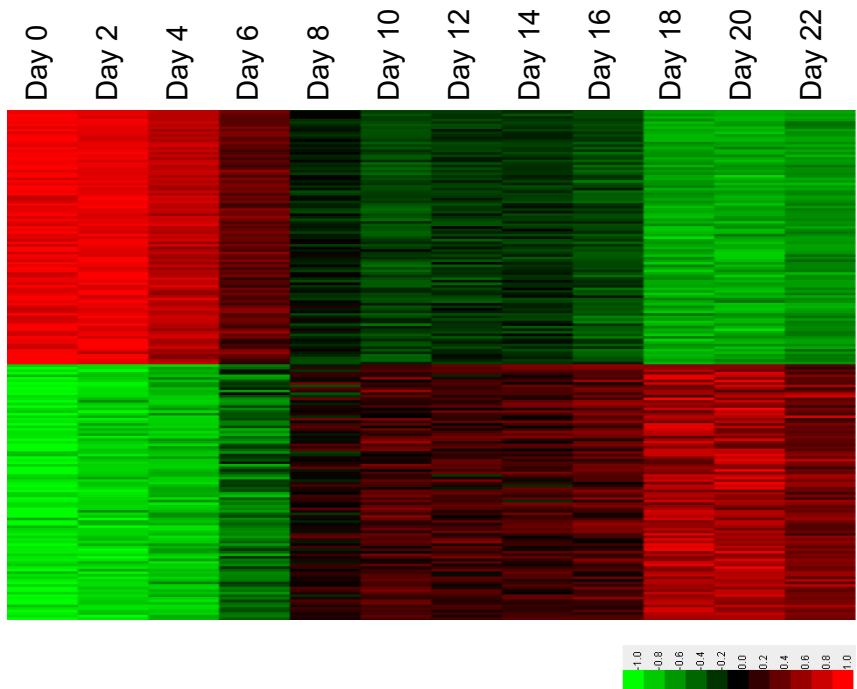


Figure S3

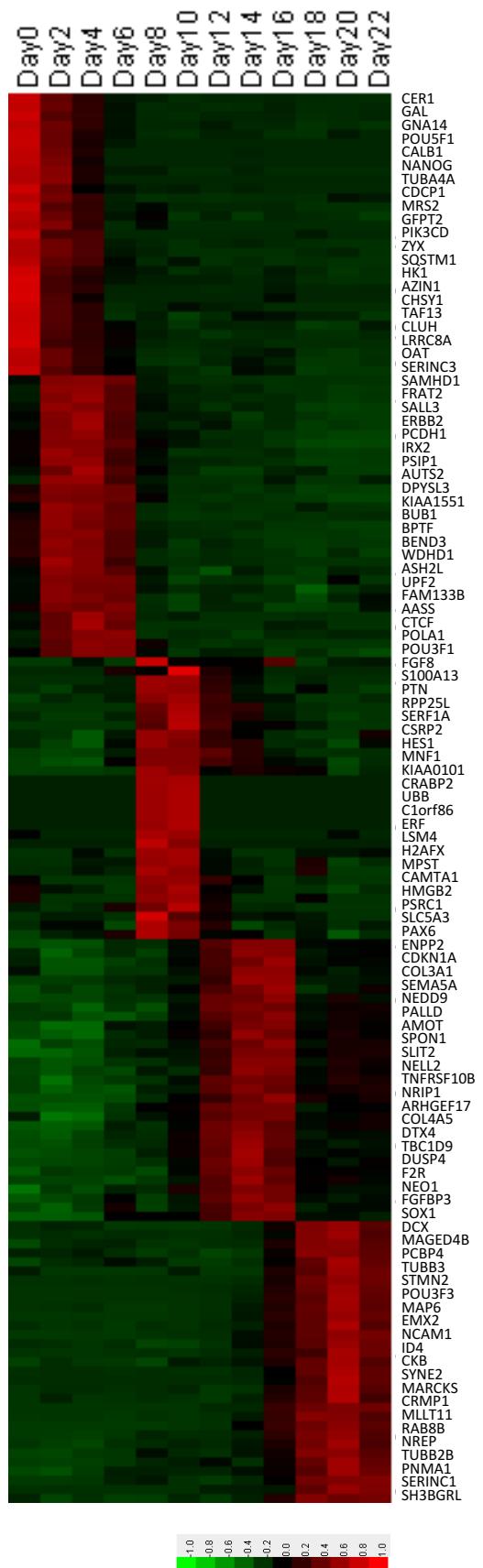
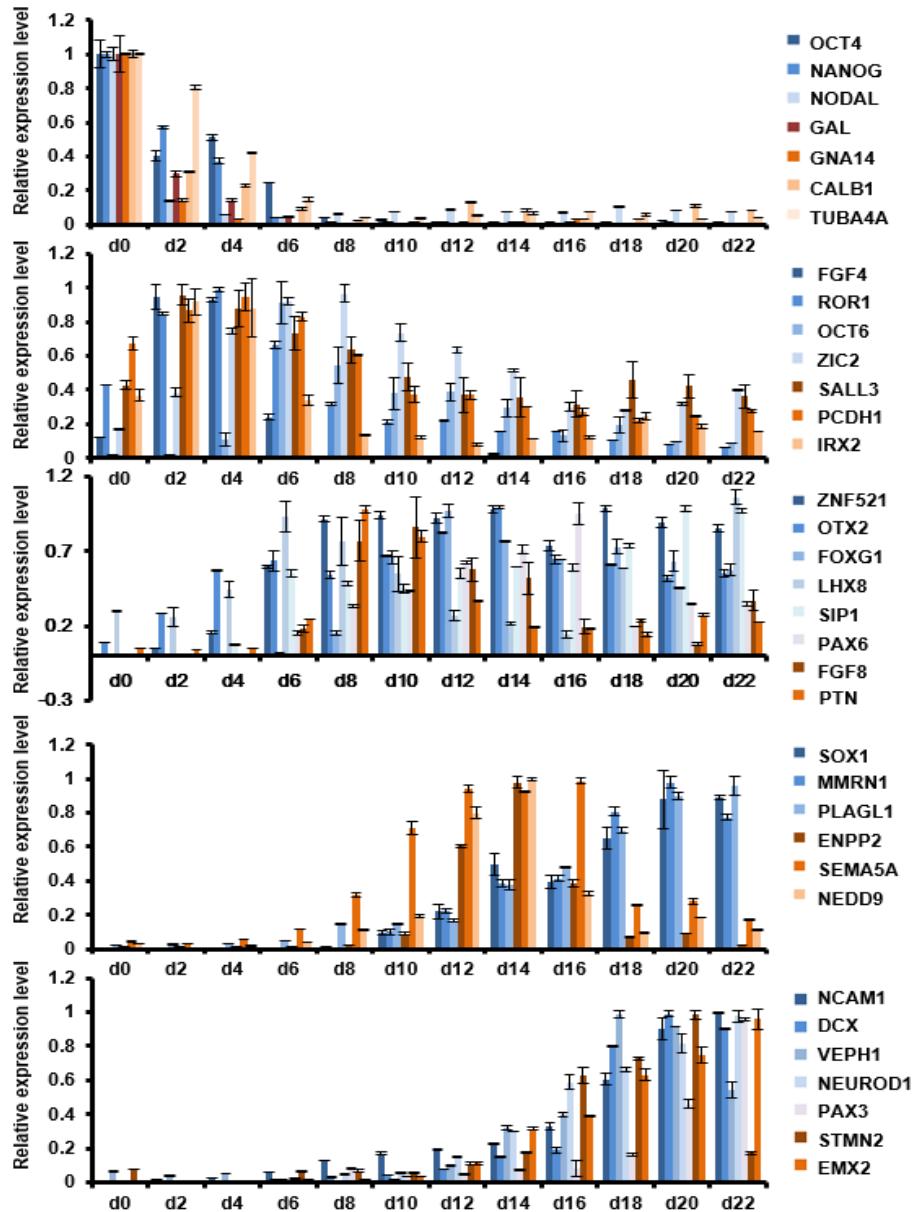


Figure S4

A



B

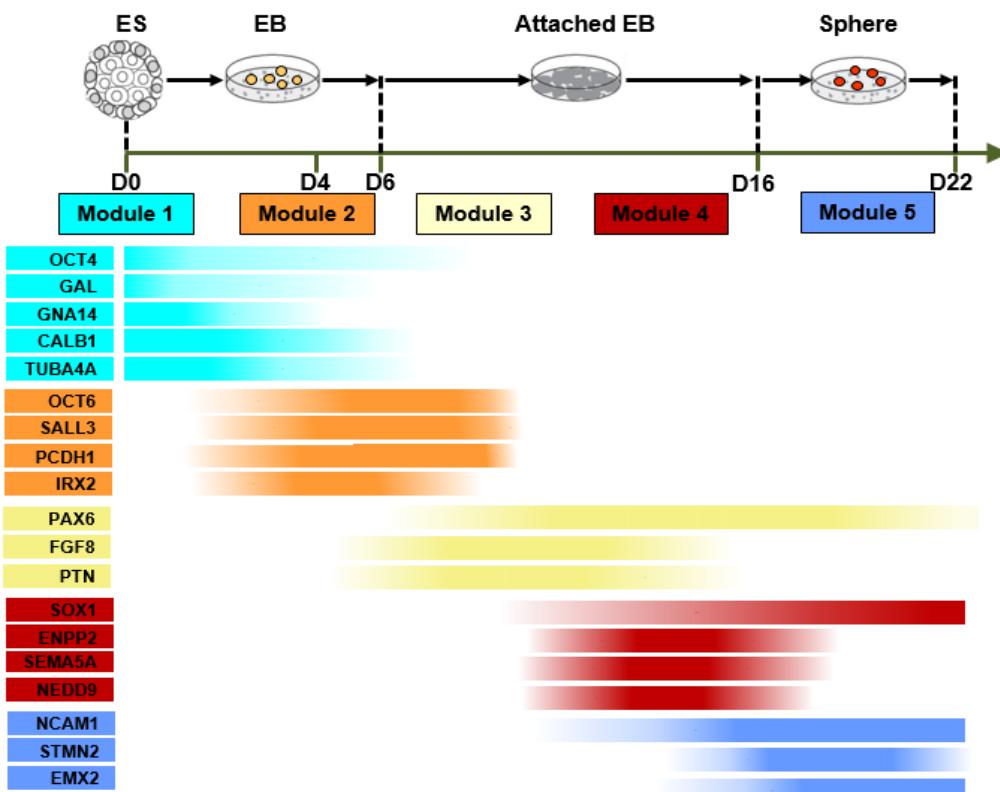


Figure S5

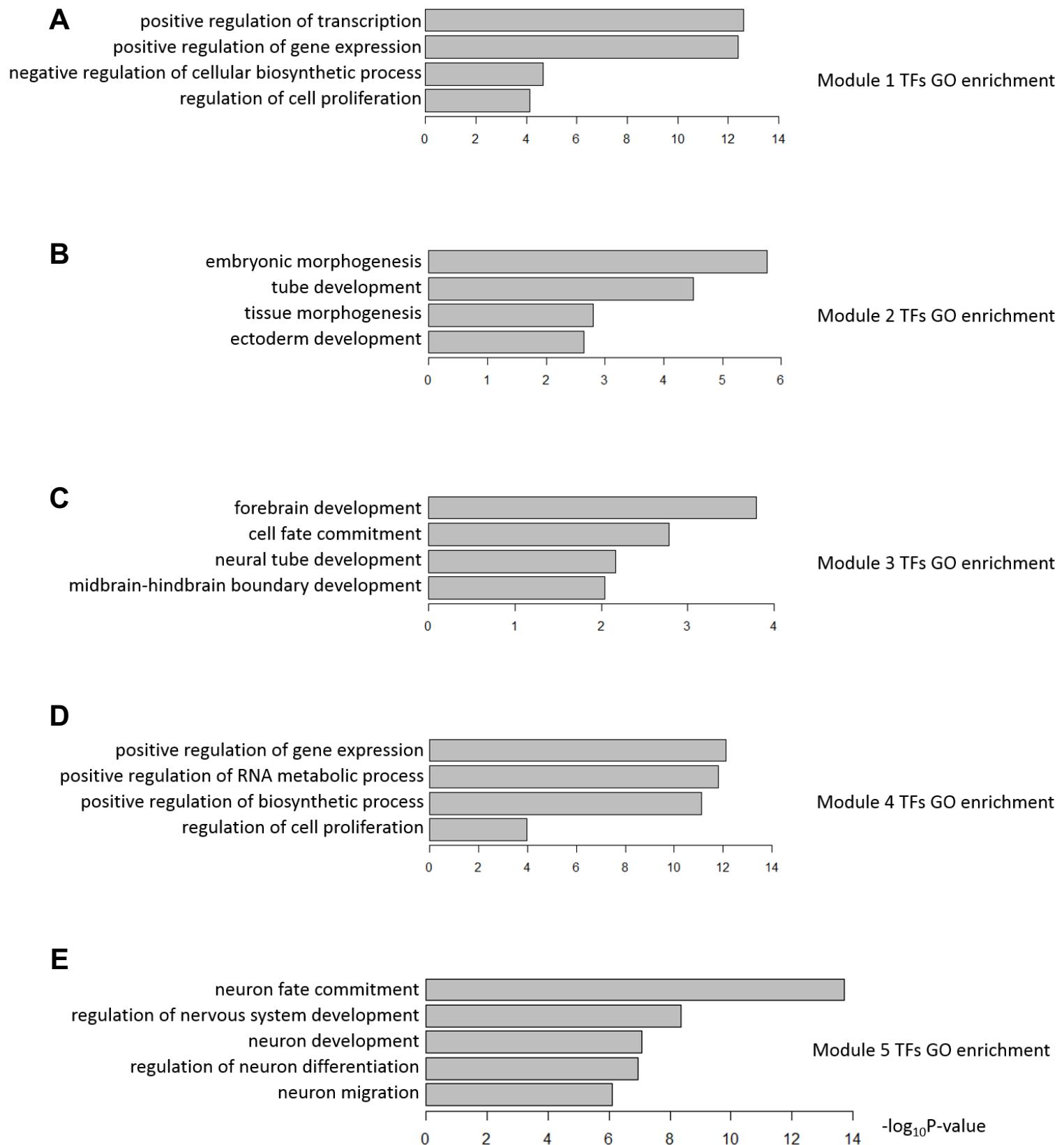
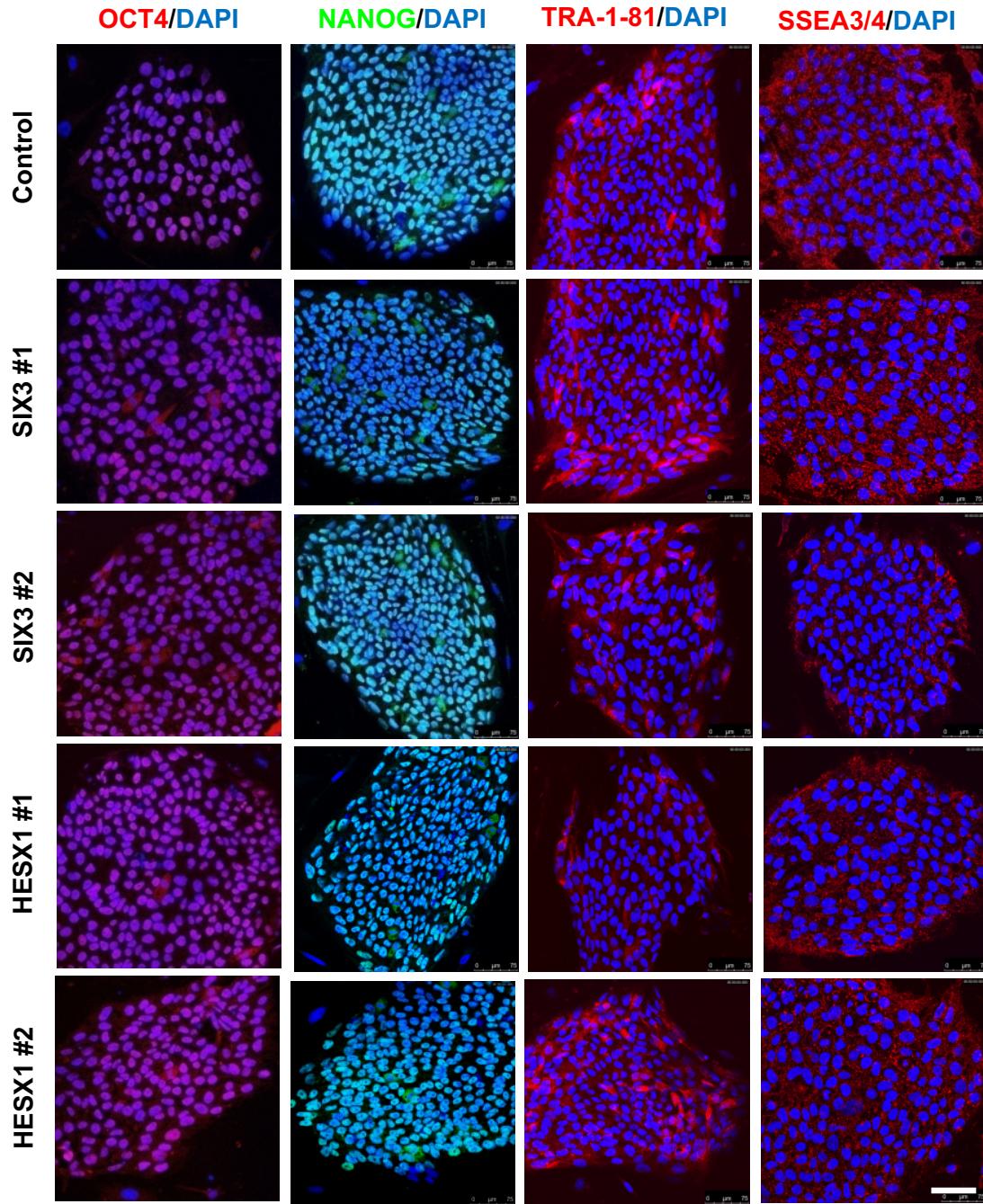


Figure S6

A



B

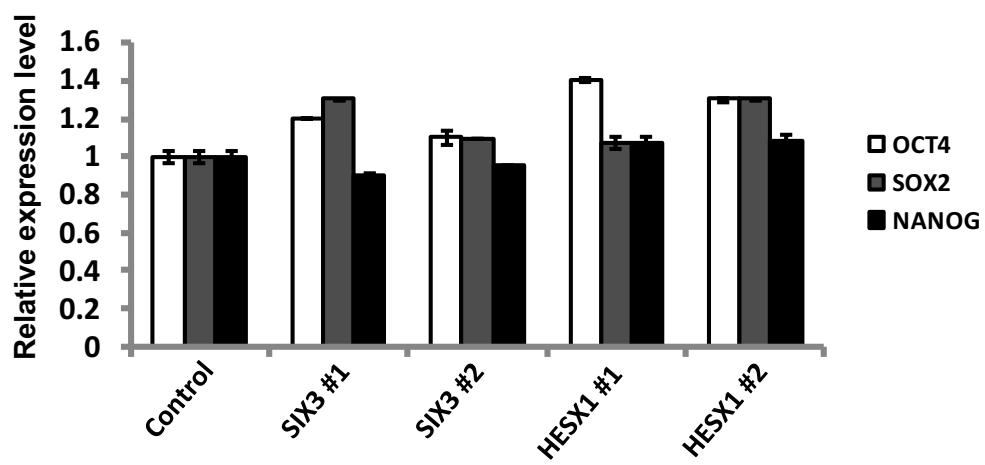
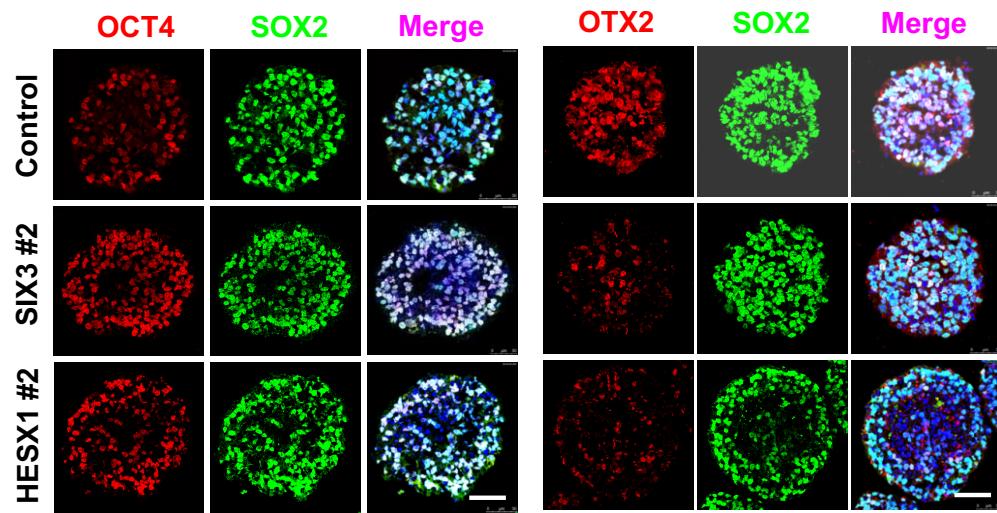
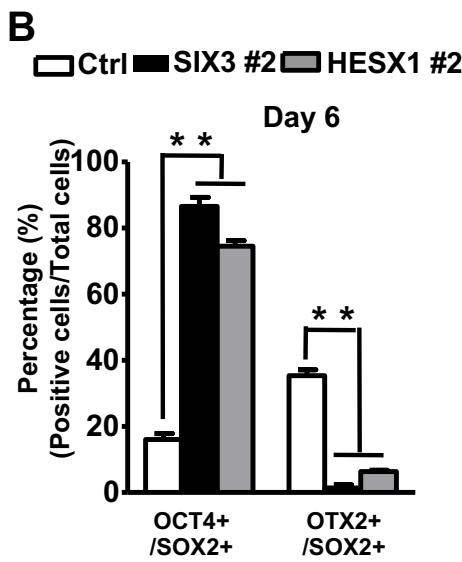
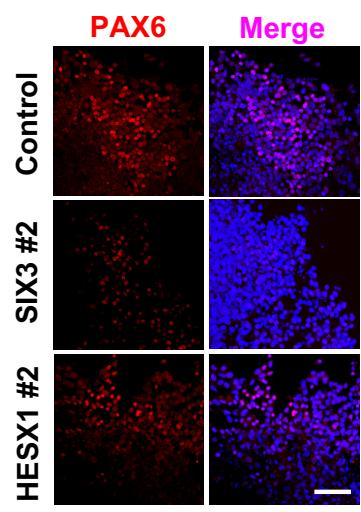
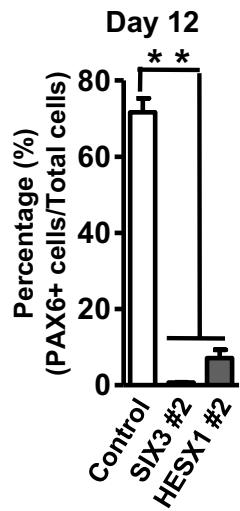
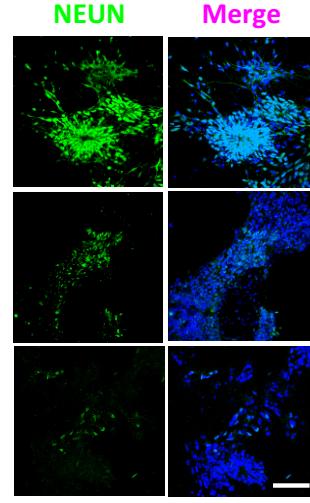
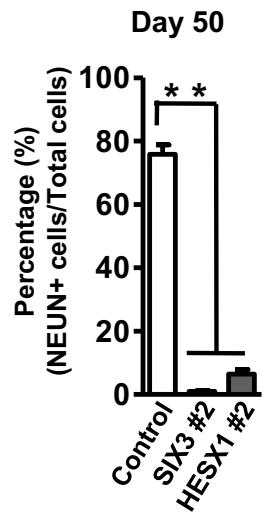


Figure S7**A****B****C****D****E****F**

Supplemental Table 1

Transcription factors in each module

Modules	Transcription factors
1 (Day 0)	ARNTL2, BCL11A, BHLHE40, BNC1, CSDC2, ELF4, ELK3, ETS1, ETS2, ETV4, FLI1, FOSL1, FOSL2, FOXC2, FOXD3, FOXH1, FOXN3, FOXO1, HEY2, HMOX2, IRF6, JUN, KLF10, KLF5, KLF7, LHX4, MYC, N4BP3, NANOG, NFE2L3, NR3C1, POU2F2, POU2F3, POU5F1, PRDM14, RREB1, RUNX1, SIX4, SMAD7, SP140, SP140L, TEAD4, TFAP2C, TFCP2L1, TGIF1, TSHZ3, TWIST1, XBP1, ZFP42, ZIC3, ZSCAN10
2 (Day 2, 4, 6)	ARID2, BCL6B, BPTF, CTCF, ELF3, FOXB1, FOXO4, GLI1, GRHL3, HES3, HHEX, ID1, IRX2, KLF4, LHX5, LIN28A, MYB, MYCN, NFE2, NR1D2, NR6A1, PBX1, RARG, REST, SALL4, TFAP2A, YBX2, ZIC2, ZIC5, ZNF202, ZNF217, ZNF296, ZNF480, ZNF607, ZNF616, ZNF644, ZNF675, ZNF678, ZNF92
3 (Day 8, 10)	CAMTA1, EDF1, ERF, GBX2, HAND1, HES1, HESX1, ID2, ID3, LZTS2, MBD3, MRPL28, PAX2, PAX6, PAX8, PBX2, POU3F1, PRRX2, RAX, SIX3, SIX6, SLC2A4RG, USF2, VPS72, ZNF460, ZNF771
4 (Day 12, 14, 16)	AHR, ATF3, ATF6, CREB3L2, DBX2, EPAS1, FOSB, FOXE3, FOXP4, GLIS3, IKZF2, IKZF3, KLF3, MAF, MAFF, MITF, MYRF, NFE2L2, PPARA, RARB, SMAD3, SMAD9, SNAI2, TBX2, TCF12, TEAD1, TP63, VSX2, ZBTB16
5 (Day 18, 20, 22)	ARNT2, ARX, ASCL1, BACH2, BARHL2, BARX1, BAZ1A, CREB5, DDIT3, DLX1, DLX2, DLX5, DMRT3, DMRTA1, EGR1, EOMES, ESRRG, FEZF2, FOS, FOXG1, FOXJ1, FOXN4, FOXP2, GLI4, GSX2, HES4, HES5, HES6, HEY1, HMX1, ID4, INSM1, INSM2, ISL1, LHX2, LHX8, LMX1A, LZTS1, MECOM, MEF2C, MEIS1, MEIS2, MEIS3, MSX1, MXD4, MXI1, MYT1, MYT1L, MZF1, NEUROG1, NEUROG2, NHLH1, NKX2-1, NKX2-3, NKX6-1, NPAS3, NR2E1, NR2F1, NR2F2, OLIG1, OLIG2, ONECUT1, ONECUT3, OTX1, PAX3, PLAGL1, POU3F2, POU3F3, POU3F4, POU6F1, PRDM16, RFX2, RFX4, RORB, RXRB, SCRT2, SP8, ST18, TAL2, TBR1, TRPS1, TSHZ1, UNCX, VAX1, VAX2, ZBTB20, ZEB1, ZEB2, ZFHX3, ZFP30, ZIC1, ZIC4, ZKSCAN1, ZMAT1, ZNF25, ZNF250, ZNF324, ZNF333, ZNF436, ZNF441, ZNF467, ZNF491, ZNF497, ZNF524, ZNF536, ZNF540, ZNF584, ZNF610, ZNF688, ZNF704, ZNF821, ZNF84, ZSCAN1, ZSCAN18

Supplemental Table 2

SgRNA sequence

SgRNA	Sequence
HESX1 SgRNA-fwd	CACCGGCCTGTGGGGTTTCATTAA
HESX1 SgRNA-rev	AAACTTAATGAAACCCCCACAGGCC
SIX3 SgRNA-fwd	CACCGTGGTGAGAATCGCGAAGTT
SIX3 SgRNA-rev	AAACAACTTCGCCGATTCTCACAC
Scramble SgRNA-fwd	CACCGCTGATCTATCGCGGTCGTC
Scramble SgRNA-rev	AAACGACGACCGCGATAGATCAG

Supplemental Table 3 : Antibodies

Antibody	Isotype	Source	Identifier
OCT4	Mouse IgG	Santa Cruz	Cat#sc-5279
SOX2	Rabbit IgG	Abcam	Cat#ab59776
NANOG	Rabbit IgG	CST	Cat#8822
TRA-1-81	Mouse IgG	Chemicon & Millipore	Cat#90233
SSEA3/4	Mouse IgG	Chemicon & Millipore	Cat#90231
SIX3	Mouse IgG	Abcam	Cat#AB172131
HESX1	Rabbit IgG	Abclonal	Cat#A10696
GAPDH	Mouse IgG	Abclonal	Cat#AC002
PAX6	Rabbit IgG	Covance	Cat#AB2237
NESTIN	Mouse IgG	Abcam	Cat#AB6142
OTX2	Goat IgG	R&D system	Cat#AF1979
SOX1	Mouse IgG	R&D system	Cat#AF3369
MAP2	Mouse IgG	Sigma	Cat#M4403
NEUN	Rabbit IgG	Chemicon & Millipore	Cat#ABN78
VGLUT1/2	Mouse IgG	Chemicon & Millipore	Cat#MAB5502
GAD67	Rabbit IgG	Chemicon & Millipore	Cat#AB1511
TH	Rabbit IgG	Chemicon & Millipore	Cat#657012
VACHT	Rabbit IgG	Synaptic systems	Cat#139103
TBR1	Rabbit IgG	Abcam	Cat#AB31940